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ACCENTUATED SELECTIVE POPULATION TRANSFER DIFFERENCE SPECTROSCOPY: A NEW METHOD FOR REVEALING HIDDEN PROTON NMR RESONANCES.

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Summary: Block difference FID accumulation with an initial selective decoupler pulse alternately placed at the high and low frequency lines of a multiplet provides, upon transform, a difference spectrum that shows only those other multiplets that are scalar coupled to the probed resonance with full retention of all splittings for the multiplets so revealed.

High-field programmable multipulse FT-NMR instruments are now routinely available for structure elucidation of moderately complex natural products (MW 300-800). If obtaining a full complement of 2D spectra[†] is precluded due to either instrument time constraints or compound instability, the major obstacle in the spectral assignment process is locating (and visualizing the multiplicity of) individual resonances in crowded spectral regions. Two techniques -- decoupling difference and ΔNOE spectra -- have been advocated[3] for revealing hidden multiplets that display either scalar or dipolar coupling to resonances in less crowded regions. Both of these methods suffer from significant subtraction error problems[†] that can be only partially corrected[4]. The correction routines preclude direct accumulation of a difference FID and require software that is not routinely available. In ΔNOE spectroscopy an additional complication appears. Imprecise placement of the on-resonance decoupler pulse (or the use of low power settings to obtain selectivity) results in differential saturation of lines within a multiplet and superimposes selective population transfer (SPT) effects upon the ΔNOE spectra. The supression of SPT effects requires careful selection of composite pulses in the selective pre-irradiation period.[5]

We now report that the reverse of the SPT suppression procedure of Neuhaus[5] provides a powerful difference technique for uncovering hidden resonances in a multiplet form which retains all of the scalar coupling constants. The method (outlined in the summary, detailed in a later section) provides information equivalent to that of PS-COSY[2] and bears an obvious relationship to the FT-INDOR experiment.[6] The effects of SPT in difference spectroscopy can be illustrated by the changes in the A portion of an ABXY spectrum (J_{AB}, J_{AX}, J_{BY} positive; $J_{BX}\approx 0$) upon selective perturbation of lines within the X multiplet, see **Figure 1**.

[†] A representative set of 2D spectra[1] would be: COSY, NOESY, and 2D-J. 2D-J spectroscopy reveals (except in the case of $J_{ij}>\Delta\nu_{ij}$) the individual multiplet patterns from overlapping regions, but cannot provide specific connectivities. COSY spectra define scalar connectivities, but in the absence of high resolution, 1K by 4K real points or greater, phase sensitive (PS) detection coupling constants cannot be extracted from COSY plots. The latter require prohibitive instrument and computer time; a published[2] PS-COSY (1K x 4K point) for a 16 mM protein solution required 40 h for data accumulation and 10 h of transform time for the 2ppm width of the amide NH region after zero-filling to 0.4 Hz/pt digital resolution in ω_2.

[†] In decoupling difference spectra, signals are of necessity acquired with the decoupler on a different frequency for the differenced blocks. The resulting Bloch-Siegert shifts produce difference signals that can be confused with the expectations for scalar decoupling. In ΔNOE spectra the desired signals are frequently small (0.2-5% of $I_z^{control}$) and the source of the common "dispersion" signal artifacts, phase shifts *versus* frequency shifts, is apparently unknown[4].

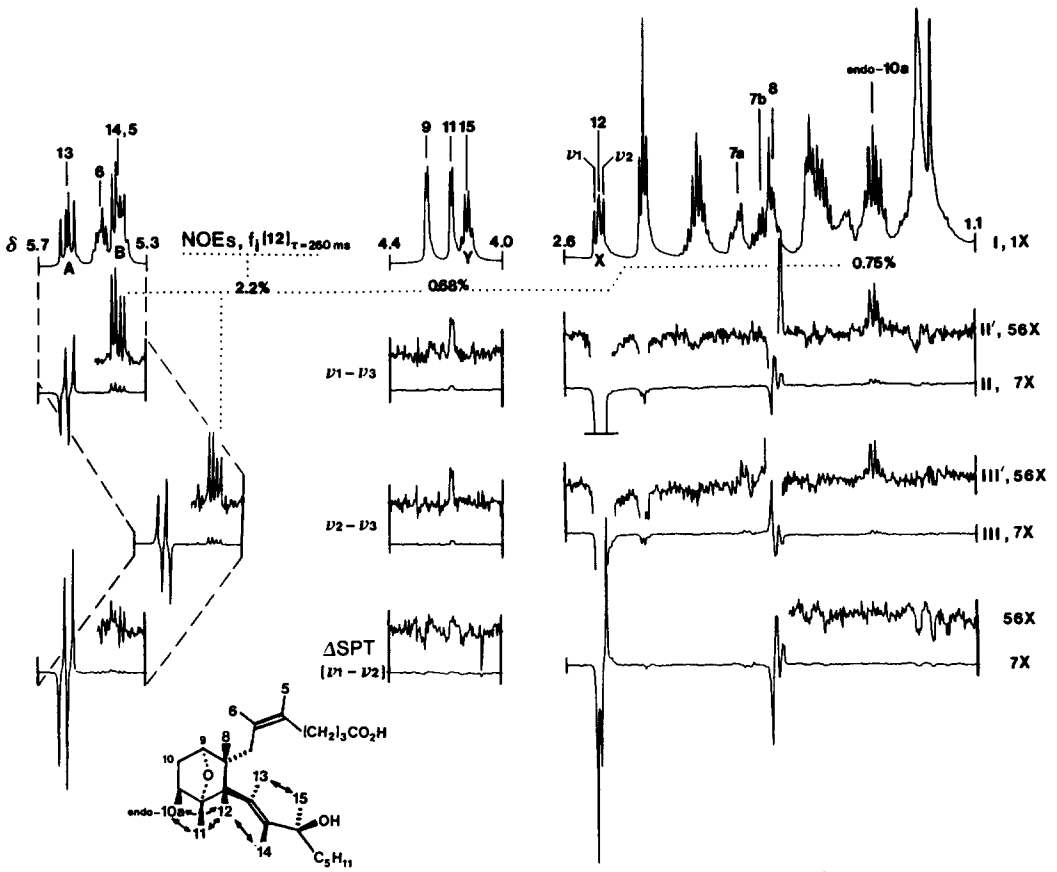


Figure 1. Comparisons of control (I), off-center ΔNOE (II and III), and ΔSPT spectra for 20 mM SQ-26536 (in $CDCl_3$). Trace II shows a ΔNOE spectrum for a transient[8] experiment (τ = 260 ms) with the selective "on-resonance" pulse ($\theta \approx 105°$) centered on H-12 line ν1. Trace III utilized ν2 in the "on-cycle"; both employ ν3 (baseline at 3 ppm) as the "off-cycle" control.

The structural inset of Figure 1 shows SQ-26536, a potent antagonist of platelet aggregators,[7] with arrows indicating spatial proximities noted in ΔNOE spectra. The SPT effects on H-13 and H-8 line intensities are clearly larger than the NOEs observed at H-14, 11, and *endo* 10α. When the on-resonance pulse center is moved from the high to low frequency portion of the multiplet the signs of all the SPT signals are reversed. The accentuated SPT difference spectrum (pulse sequence as in Methods Description) corresponds to (II - III) and reveals only the scalar coupled resonances, with dipolar NOEs completely suppressed.

The remaining examples of the application of accentuated SPT differencing are taken from our studies of $PGF_2\alpha$ in dilute D_2O buffer[9]. The spectral region from 1.5-1.7 ppm contains severely overlapping multiplets from six protons (H-3a,3b,8,10α,16a, and 16b); and prior to our use of ΔSPT complete assignment and extraction of all J-values required in excess of 40 hr of instrument time. In striking contrast, individual ΔSPT experiments typically require only several minutes for ΔFID acquisition[10]. The traces in Figure 2 illustrate the use of ΔSPT to assign connectivity and to excise individual multiplets from the severely crowded spectral region. The clean difference signal for H-8, a broad complex multiplet hidden under the C-3 methylene quintet and half of the C-16 methylene AB of $ABXY_2$, is particularly noteworthy.[11]

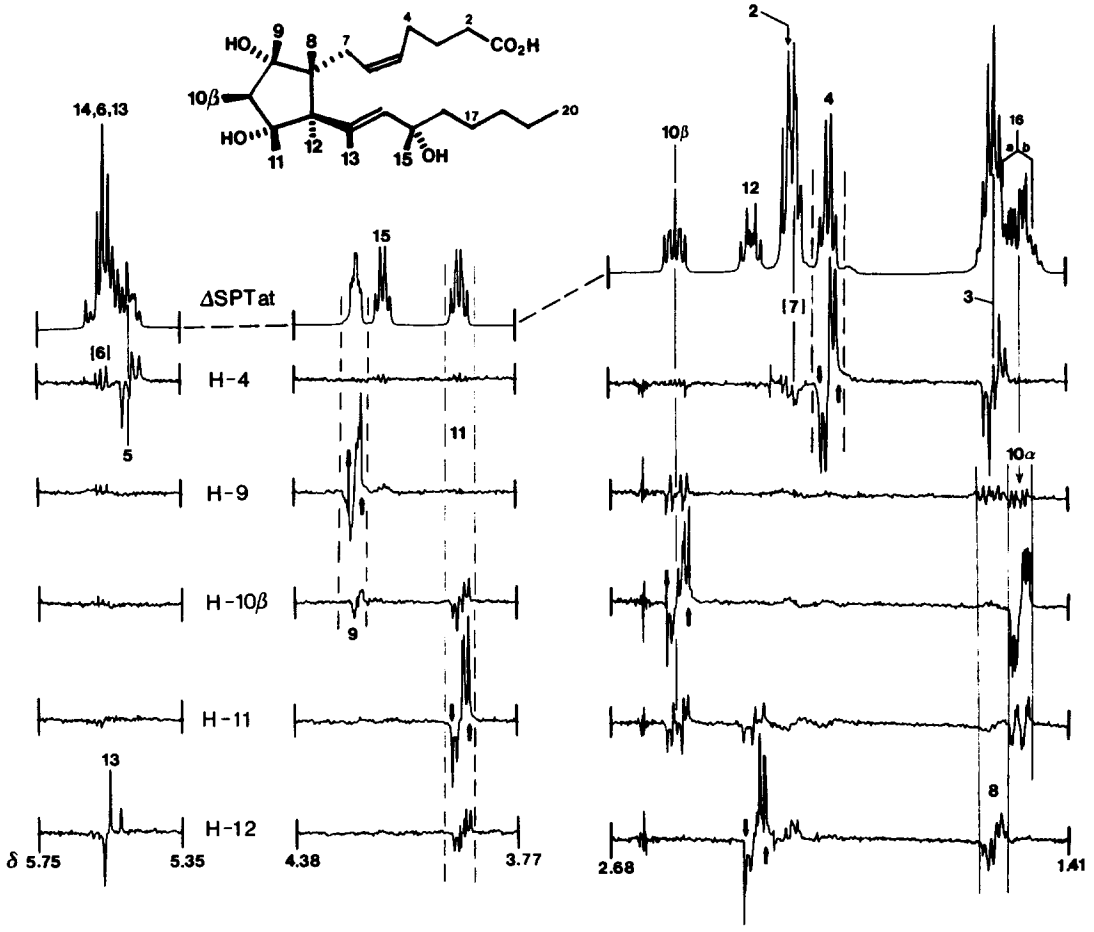


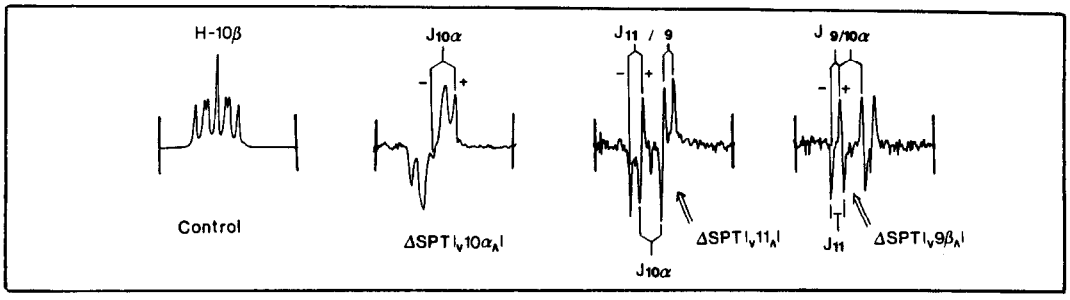
Figure 2. Accentuated ΔSPT spectra for 10 mM $PGF_2\alpha$ (in 20 mM K_2DPO_4/D_2O) appear below the control spectrum. Arrows (↑,↓) indicate the "on" and "off" resonance positions employed.

Methods Description. In order to facilitate ready applicability in other laboratories we have used a modification of the standard pulse program for direct accumulation of a difference FID from small blocks of data at different decoupler frequencies:

$$\{[PD-{}^{\nu 1}\theta(PW{=}t_1)-\tau-{}^{ns}\theta\ AQ\ FID(t_2)]_8\ MINUS\ [PD-{}^{\nu 2}\theta(PW{=}t_1)-\tau-{}^{ns}\theta\ AQFID(t_2)]_8\}_n$$

with -- PD = preparatory delay; ${}^{s}\theta$ = a selective perturbation, flip angle = θ, of pulse width t_1 centered at $\nu 1$, $\nu 2$, etc.; τ = mixing time; and ${}^{ns}\theta$ is a non-selective acquisition pulse of flip angle equal to or less than the Ernst angle - $\cos^{-1}[\exp-(t_2+t_1+PD+\tau)/T_1]$. ΔNOE spectra[8] are generated with $\nu 2$ "off-resonance" to produce, in the second block, a control spectrum for subtraction. Complete NOE suppression with SPT accentuation is accomplished by placing ${}^{\nu 1}\theta$ at the highest frequency line of the probing multiplet while the "off-resonance" pulse is replaced by one located at the lowest frequency line of the same multiplet. A moderate decoupler power ($\gamma B_2/2\pi$ = 2.5-10 Hz) is chosen so that the extreme states, ($\alpha\alpha$...) *versus* ($\beta\beta$...), are saturated with partial selectivity while a 40-60 ms PW gives an average 80-130° flip angle over the entire multiplet. "Mixing times" of 100 ms or less are used when only the scalar-related SPT effects are to be observed.[12]

The specific parameters for the experiments[13] in Figures 1 and 2 were: Fig. 1 -- PD = 1.7 s, t_1 = 40 ms (DP = 38L), τ = 260 ms, ${}^{ns}\theta \approx 47°$ (PW = 6.5 μs), t_2 = 820 ms, n=48; Fig. 2 -- PD = 1.3 s, t_1 = 40 ms (DP = 50L), τ = 60 ms, ${}^{ns}\theta \approx 72°$ (PW = 10.2 μs), t_2 = 2.04 s; n = 32, all ΔSPT spectra at the same attenuation.



The traces immediately above show the 10β-H multiplet of PGF$_2\alpha$ and provide an illustrative example of how J values can be derived by this technique. (SPT effects are also seen for strongly coupled spin systems, but their quantitative interpretation is more complicated). Accentuated SPT differencing is clearly a simple, and rapid, technique for unmasking the identity and multiplicity of hidden proton resonances. We anticipate that the method will find wide applicability in NMR assignment and structure elucidation.

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References and Notes

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7. P.W.Sprague, J.E.Heikes, D.N.Harris and R.Greenberg, *Adv. Prostaglandin Thromboxane and Leukotriene Res* 11, 337-343 (1983).

8. In steady-state NOEs ($t_1 \gtrsim 3$ s, $\tau \lesssim 10$ ms), for transient NOEs ($t_1 \lesssim 100$ ms, and τ is varied).

9. N.H.Andersen, B-S.Lin, D-J.Liu and C.H.Wilson, "High Field NMR Probes of the Media Dependent Conformational Changes of Prostaglandins" in *Vth Internat'l. Prostaglandins Conf. Abstract Book* (Florence, Italy), p. 687 (1982); N.H.Andersen and B-S.Lin, manuscript submitted to *Biochemistry*.

10. Qualitative data suitable for assignment purposes can be obtained with 4-8 blocks of eight using PD = 1.1-2 s in the pulse sequence of the Methods Description. The illustrated traces are taken from 16-48 cycle experiments (10-30 min) in order to display better S/N, minimize subtraction errors, and reveal the much smaller NOEs. In this difference method there is no reason to use extensive relaxation delays.

11. The only fully successful visualization of the H-8 multiplet required the use of an inversion recovery sequence on 3,3,4,4-d$_4$-PGF$_2\alpha$ using 2500 transients (5.5 h) due to the similar T_1 values for H-8,16a, and 16b.

12. The post-decoupler pulse delay is not properly a "mixing time". The transmitted SPT effects would be maximal at τ=0 and with fully selective inversion (or saturation) of the extreme lines, however the selectivity requirement would necessitate very low decoupler power settings ($\lesssim$1 Hz) and long driving times during which dipolar NOEs would develop. In practice, sufficient selectivity with t_1 < 60 ms results only with effective flip angles significantly less than 180°. When $\theta \lesssim 120°$, the SPT effects are maximized, both in absolute terms and relative to the NOEs, by using a very small acquisition flip angle. The considerations involved in designing an optimal set of pulses and τ-value will be detailed in the full report of this work.

13. All spectra illustrated were obtained on the Bruker WM-500 using quadrature detection with an eight step phase cycle — x,x,-x,-x,y,y,-y,-y — using interleaved blocks of eight. The decoupler power settings (low power, DP = 38-50L) correspond to those used in selective NOE experiments ($\gamma B_2/2\pi$ = 9-2.5 Hz).

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